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# Fluorophore−Quencher Based Activatable Targeted Optical Probes for Detecting *in Vivo* Cancer Metastases

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**Abstract:** *In vivo* molecularly targeted fluorescence imaging of tumors has been proposed as a strategy for improving cancer detection and management. Activatable fluorophores, which increased their fluorescence by 10-fold after binding tumor cells, result in much higher target to background ratios than conventional fluorophores. We developed an *in vivo* targeted activatable optical imaging probe based on a fluorophore−quencher pair, bound to a targeting moiety. With this system, fluorescence is quenched by the fluorophore−quencher interaction outside cancer cells, but is activated within the target cells by dissociation of the fluorophore−quencher pair. We selected the TAMRA (fluorophore)−QSY7 (quencher) pair and conjugated it to either avidin (targeting the D-galactose receptor) or trastuzumab (a monoclonal antibody against the human epithelial growth factor receptor type2 (HER2/neu)) and evaluated their performance in mouse models of cancer. Two probes, TAMRA−QSY7 conjugated avidin (Av-TM-Q7) and trastuzumab (Traz-TM-Q7) were synthesized. Both demonstrated better than similar self-quenching probes. *In vitro* fluorescence microscopic studies of SHIN3 and NIH/3T3/HER2+ cells demonstrated that Av-TM-Q7 and Traz-TM-Q7 produced high intracellular fluorescent signal. *In vivo* imaging with Av-TM-Q7 and Traz-TM-Q7 in mice enabled the detection of small tumors. This molecular imaging probe, based on a fluorophore−quencher pair conjugated to a targeting ligand, successfully detected tumors *in vivo* due to its high activation ratio and low background signal. Thus, these activatable probes, based on the fluorophore−quencher system, hold promise clinically for "see and treat" strategies of cancer management.

**Keywords:** Molecular imaging; FRET; photoquencher; activatable; cancer

## Introduction

Molecular imaging based on optical fluorophores will allow physicians to "see and treat" pathology more effectively during surgical and endoscopic procedures. High target (tumor)-to-background ratios (TBR) improve both the sensitivity and specificity of optical imaging. One method of improving TBR is to activate the targeted fluorophore only after it has bound to the intended target. In this manner, the fluorescent signal can be turned "on" only at the target tumor. Several different classes of activatable optical probes have been reported for *in vivo* imaging including self-quenching systems[1−4] and photon-induced electron transfer based fluorophores.[5,6] Herein, we propose another class of acti-

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vatable optical probes for *in vivo* imaging using the fluorophore−quencher interaction which is thought to be based on fluorescence resonance energy transfer (FRET) quenching.

FRET is a mechanism, reported by Förster in 1948, by which energy is transferred from an electronically excited donor molecule to a ground-state acceptor molecule.[7] If the acceptor molecule does not fluoresce, the donor fluorescence is diminished by FRET, as long as the acceptor is less than 1.8 × the Förster distance, typically less than 100 Å, from the donor.[8] If the acceptor is released from its steric proximity to the donor, then FRET quenching is abolished and the donor fluorophore is activated. FRET-based activation has been used *in vitro* for DNA biosensors and investigation of protein conformation change.[9−11] Furthermore, intravital microscopy based on FRET in living animals is also commonly used.[12]

Here, we propose a novel, activatable optical probe for *in vivo* imaging based on the FRET quenching mechanism. That is, the fluorescence is quenched by the fluorophore−quencher interaction when the probe is outside the target cells, but is activated after cellular internalization when the fluorophore and its quencher are disassociated in the endosome/lysosome (Figure 1). The advantage of this system over conjugates based on the self-quenching (homo-FRET) mechanism is that much higher quenching efficiency is achieved than with other conventional mechanisms including self-quenching employing multiple conjugations of Rhodamine X or Cy 5.5 fluorophores[3,4] leading to reduced background fluorescence.

Rhodamine-core-derived fluorophores offer several desirable properties including good photostability, high extinction coefficients, and high fluorescence quantum yield. Recently, the rhodamine-core fluorophore, TAMRA, has been shown to have superior characteristics for *in vivo* fluorescence imaging compared to other members of the rhodamine family because it is intrinsically activatable after cellular internalization.[13] This intrinsic activation mechanism can be combined with other quenching mechanisms to generate higher activatable capacity than is possible with other fluorophores.

When TAMRA is in close proximity to the acceptor QSY7, an effective FRET donor−acceptor pair is created with an overlap of the emission spectra of TAMRA and absorption spectra of QSY7 of more than 40%. TAMRA−QSY7 pairs, which have been conjugated to peptides, have been used in *in vitro* assays of protease activity.[14] However, the *in vivo* potential of such FRET pairs has not been fully explored. Therefore, we designed molecular imaging probes based on the TAMRA−QSY7 fluorophore−quencher pair for *in vivo* imaging of two different cancers targeting a D-galactose receptor (a H-type lectin) and a HER2/*neu* cell surface antigen, and evaluated their efficacy for *in vivo* tumor detection.

## Materials and Methods

**Reagents.** Avidin was purchased from Pierce Biochemical, Inc. (Milwaukee, WI). Trastuzumab, an FDA-approved humanized anti-HER-2 antibody, which has a complimentary determination region (CDR) against HER-2 grafted on a human IgG1 framework, was purchased from Genentech Inc. (South San Francisco, CA). Daclizumab, a murine-human chimerized mAb to the IL-2Rα (Tac) receptor of T-cells, was purchased from Hoffmann-La Roche Inc. (Nutley, NJ). TAMRA-NHS ester and QSY7-NHS ester were purchased from Invitrogen Corporation (Carlsbad, CA). ZsGreen plasmid was purchased from Clontech Laboratories, Inc. (Mountain View, CA). All other chemicals used were of reagent grade.

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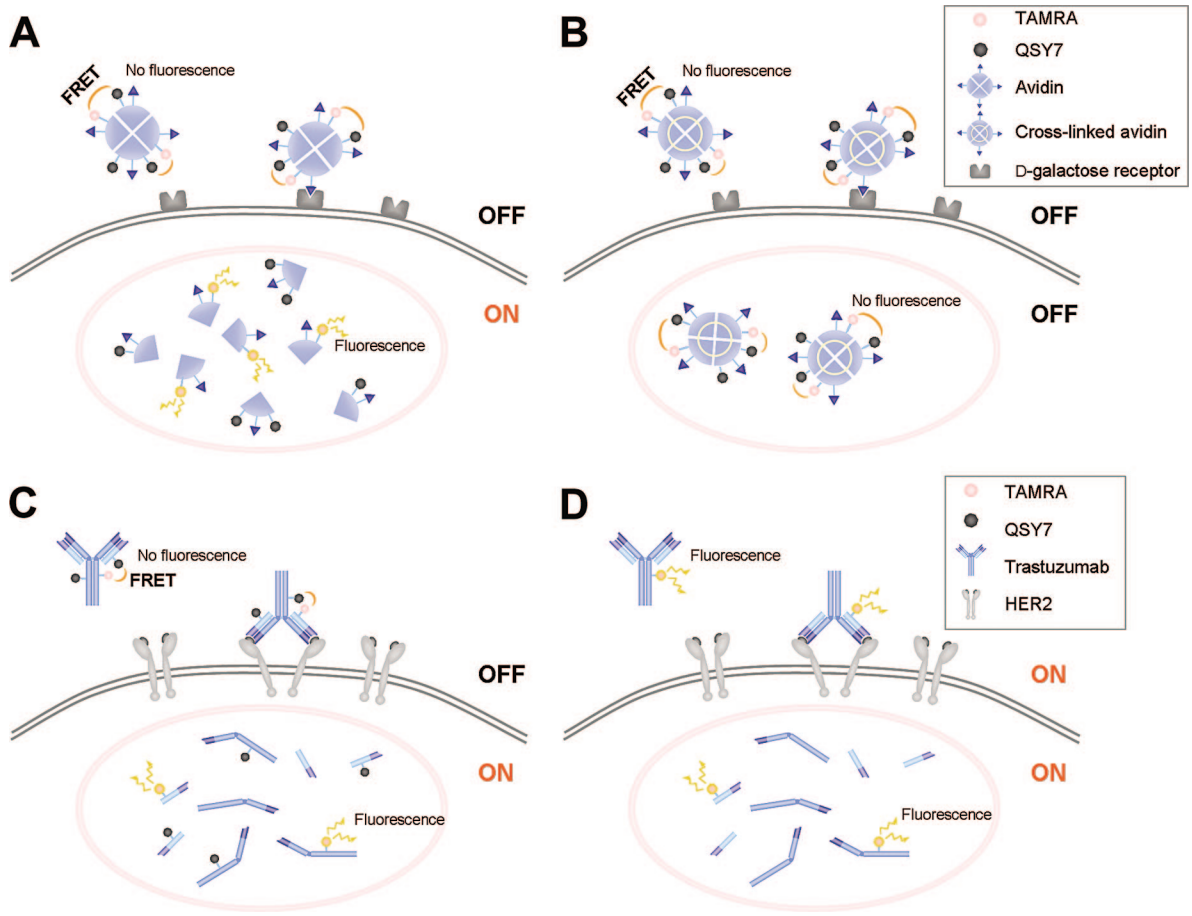


***Figure 1.*** Schematic illustration of the concept of activatable probe with FRET. (A) The "activatable" probe, Av-TM-Q7, is quenched by FRET outside of the cell. When it binds to D-galactose receptor and is internalized, it is catabolized into monomers within the endoplasmic vesicles and becomes fluorescently activated by dequenching, with strong fluorescence signal within the cells. (B) If the catabolism is blocked by cross-linking, FRET quenching continues and the Av-TM-Q7 cannot be activated. (C) The "activatable" probe, Traz-TM-Q7, is quenched by FRET outside of the cell. When it binds to HER2 and is internalized, it is catabolized within the cell and FRET quenching is abolished. It is fluorescently activated by dequenching, and a strong fluorescence signal is found within the cells. (D) The "always-on" probe, Traz-TM, is active both outside of the cell and inside of the cell reducing the target to background ratio.

**Synthesis of TAMRA, QSY7 Conjugated Avidin (Av-TM-Q7).** Avidin (0.8 mg, 12 nmol) was incubated with TAMRA-NHS (32 $\mu$g, 60 nmol, 15 mM in DMSO) in 0.1 M $Na_2HPO_4$ (pH 8.5) at room temperature for 30 min. The mixture was purified with a Sephadex G50 column (PD-10; GE Healthcare) and concentrated using a centrifugal filter device (Centricon YM-30, Millipore, Bedford, MA) to 37 $\mu$M. Then, QSY7-NHS (88 $\mu$g, 112 nmol, 10 mM in DMSO) was added to this solution and reacted in 0.1 M $Na_2HPO_4$ (pH 8.5) at room temperature for 30 min followed by the purification with a Sephadex G50 column. The resulting compound, Av-TM-Q7, was kept at 4 °C in the refrigerator as a stock solution. The protein concentration was determined with Coomassie Plus protein assay kit (Pierce Biotechnology, Rockford, IL) by measuring the absorption at 595 nm with a UV−vis system (8453 Value UV−visible Value System; Agilent Technologies, Santa Clara, CA). The concentrations of TAMRA and QSY7 were measured after the each conjugation step by absorption with the UV−vis system to confirm the number of fluorophore molecules conjugated with each avidin molecule. For accurate measurements, 5% SDS was added to the Av-TM-Q7 solution to diminish the interaction between TAMRA and QSY7 and avoid the change in absorption peak. The number of TAMRA and QSY7 per avidin was adjusted to approximately 3 and 5, respectively.

**Cross-Linking of Av-TM-Q7 (Av-TM-Q7 (CL)).** Disuccinimidyl suberate (DSS; Pierce, Rockford, IL) was used to cross-link the avidin tetramer to serve as a control conjugate that does not break down in the lysosome. Av-TM-Q7 (6 nmol) was incubated with DSS (3.6 $\mu$mol in DMSO) in 0.1 M $Na_2HPO_4$ at room temperature for 1 h.

**Synthesis of TAMRA, QSY7 Conjugated Trastuzumab (Traz-TM-Q7).** Trastuzumab (1 mg, 6.8 nmol) was incubated with QSY7-NHS (54 $\mu$g, 68 nmol, 10 mM in DMSO) in 0.1 M $Na_2HPO_4$ (pH 8.5) at room temperature for 30 min. The mixture was purified with a Sephadex G50 column (PD-10; GE Healthcare) and concentrated using a centrifugal filter device (Centricon YM-30, Millipore, Bedford, MA) to 22

nM. Then, TAMRA-NHS (34 $\mu$g, 65 nmol, 15 mM in DMSO) was added to this solution and reacted in 0.1 M $Na_2HPO_4$ (pH 8.5) at room temperature for 30 min followed by the purification with a Sephadex G50 column. The resulting product, Traz-TM-Q7, was kept at 4 °C in the refrigerator as a stock solution. The protein and QSY7 concentrations were measured by the same method as Av-TM-Q7. The amount of TAMRA, however, could not be determined because of the lack of absorption peak. To diminish the interaction between TAMRA and QSY7, the conjugate was denaturized with SDS (5%). Then the concentration of TAMRA was measured from the absorption peak and the conjugated number was calculated. The number of TAMRA and QSY7 per trastuzumab was adjusted to approximately 1 and 3, respectively.

**Synthesis of Trastuzumab TAMRA Conjugate (Traz-TM).** To investigate the quencher effect, TAMRA alone was conjugated to trastuzumab. Trastuzumab (1 mg, 6.8 nmol) was incubated with TAMRA-NHS (7.2 $\mu$g, 14 nmol, 15 mM in DMSO) in 0.1 M $Na_2HPO_4$ (pH 8.5) at room temperature for 30 min followed by purification with a Sephadex G50 column (PD-10; GE Healthcare). The resulting Traz-TM was kept at 4 °C as a stock solution. The number of TAMRA per trastuzumab was determined in the same manner as Traz-TM-Q7 concentration/number was determined, and the number of TAMRA molecules per trastuzumab was adjusted to approximately 1.

**Synthesis of TAMRA, Quencher Conjugated Daclizumab (Dac-TM-Q7) and Daclizumab TAMRA Conjugate (Dac-TM).** Dac-TM-Q7 and Dac-TM were prepared as a control to investigate nonspecific binding vs Traz-TM-Q7 and Traz-TM using the same conjugation method as shown above.

**Determination of Quenching Ability *in Vitro*.** The quenching abilities of each conjugate were investigated by denaturing with 5% SDS as described previously.[2,3] Briefly, the conjugates were incubated with 5% SDS in PBS for 10 min at room temperature. For antibody conjugates, 2-mercaptoethanol (2-ME) was also added to uncouple S−S bonds between the light and heavy chains and the mixture was heated at 95 °C for 2 min. As a control, the samples were incubated in PBS. The fluorescence signal intensity of each sample was measured with a fluorescence spectrometer (Perkin-Elmer LS55, Perkin-Elmer, Shelton, CT).

**Cell Culture.** For the investigation of the avidin conjugates, a D-galactose receptor positive ovarian cancer cell line, SHIN3, was used.[15] For the trastuzumab conjugates studies, a *HER2* gene transfected cell line and control, NIH3T3 (3T3/HER2+) and Balb/3T3/HER2− cell lines were used.[16,17] The cell lines were grown in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (Life Technologies), 0.03% L-glutamine, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin in 5% $CO_2$ at 37 °C.

**Transfection of Green Fluorescence Protein (ZsGreen).** The ZsGreen expressing plasmid was transfected into the SHIN3 and Balb/3T3/HER2− cells to validate the results with targeted fluorophores. The transfection was done with an electroporation method using Gene Plus II (Bio-Rad Laboratories, Hercules, CA). Briefly, 3 $\mu$g of ZsGreen expressing plasmid was mixed with 2 million SHIN3 cells in 400 $\mu$L of the cell culture medium (RPMI 1640 with 10% FCS). The cell suspension was then placed in a pulse cuvette (Bio-Rad Laboratories), and 250 V pulses were delivered after 950 cycles.

**Fluorescence Microscopy Studies with Avidin Conjugates.** SHIN3 cells ($1 \times 10^4$) were plated on a cover glass bottomed culture well and incubated for 16 h. Then Av-TM-Q7 or Av-TM-Q7(CL) was added to the medium (10 $\mu$g/mL), and the cells were incubated for 6 h. Cells were washed once with PBS, and fluorescence microscopy was performed using an Olympus BX51 microscope (Olympus America, Inc., Melville, NY) equipped with the following filters: excitation wavelength 530 to 585 nm, emission wavelength 605 to 680 nm. Transmitted light differential interference contrast images were also acquired.

To investigate the intracellular localization of Av-TM-Q7, colocalization studies were performed using a lysosomal marker (LysoTracker Green DND-26, Invitrogen Co., Carlsbad, CA). LysoTracker was added 30 min prior to imaging. The blue filter sets, excitation wavelength 470 to 490 nm, emission wavelength 515 to 550 nm, were additionally employed for imaging.

**Fluorescence Microscopy Studies with Antibody Conjugates.** 3T3/HER2+ cells ($1 \times 10^4$) were plated on a cover glass bottomed culture well and incubated for 16 h. Then Traz-TM-Q7, Traz-TM, Dac-TM-Q7 or Dac-TM was added to the medium (30 $\mu$g/mL), and the cells were incubated for either 1 or 8 h. Microscopy was performed in the same manner as with the avidin conjugates. To investigate the receptor specificity, a blocking study was performed by adding unlabeled trastuzumab (30 $\mu$g/mL) before Traz-TM-Q7. Colocalization studies with LysoTracker were performed in the same manner as shown above.

**Tumor Model.** All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the National Cancer Institute

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Animal Care and Use Committee. Intraperitoneal (ip) tumor implants were introduced by an ip injection of $2 \times 10^6$ SHIN3 or SHIN3 transfected with ZsGreen cells (SHIN3/ZsGreen) suspended in 300 $\mu$L of PBS in female nude mice (National Cancer Institute Animal Production Facility, Frederick, MD). The tumors grew in the mice for 3 weeks whereupon imaging was performed.

In a second animal model, metastatic lung tumors were established by intravenous (iv) injection of $2 \times 10^6$ 3T3/HER2+ cells suspended in 200 $\mu$L of PBS in female nude mice. For the two-color validation experiments, ZsGreen transfected Balb/3T3/HER2− cells (Balb/3T3/HER2−/ZsGreen) were injected into the mice from a tail vein 5 days after the 3T3/HER2+ cell injection. The experiments with the lung tumor model were performed 17 days after the 3T3/HER2+ cell injection.

***In Vivo* Spectral Fluorescence Imaging with Avidin Conjugates.** Av-TM-Q7 or cross-linked Av-TM-Q7 (50 $\mu$g/300 $\mu$L PBS) was injected into the peritoneal cavities of SHIN3 tumor bearing mice. Three hours after injection, the mice were sacrificed by carbon dioxide inhalation. The abdominal wall was then incised, and the abdominal cavity was opened to allow spectral fluorescence imaging using the Maestro In-Vivo Imaging System (CRi, Inc., Woburn, MA). Whole abdominal images and close-up peritoneal membrane images were obtained. A band-pass filter from 503 to 555 nm and a long-pass filter over 580 nm were used for excitation and emission light, respectively. The tunable emission filter was automatically stepped in 10 nm increments from 500 to 800 nm while the camera captured images at each wavelength interval with constant exposure. The spectral fluorescence images consisting of autofluorescence spectra, and the spectra from TAMRA were obtained and then unmixed based on their spectral patterns using commercial software (Maestro software CRi, Inc., Woburn, MA).

To investigate the sensitivity and specificity, Av-TM-Q7 (50 $\mu$g/300 $\mu$L PBS) was injected intraperitoneally into SHIN3/ZsGreen-bearing mice. Three hours after the Av-TM-Q7 injection, images were obtained with the Maestro In-Vivo Imaging System using two filter sets. For TAMRA fluorescence detection, a band-pass filter from 503 to 555 nm (excitation) and a long-pass filter over 580 nm (emission) were used, and for ZsGreen fluorescence detection, a band-pass filter from 445 to 490 nm (excitation) and a long-pass filter over 515 nm (emission) were used.

***In Vivo* Spectral Fluorescence Imaging with Antibody Conjugates.** Traz-TM-Q7, Traz-TM, Dac-TM-Q7 or Dac-TM (50 $\mu$g/100 $\mu$L PBS) were injected via the tail vein of mice with lung tumors arising from 3T3/HER2+ cells. One day after the injection, the mice were sacrificed with carbon dioxide. Immediately after sacrifice, the pleural cavity was exposed and spectral fluorescence images were obtained using the Maestro In-Vivo Imaging System in the same manner as avidin conjugates. The fluorescence intensities of tumor and normal lung tissue were measured in five mice on the unmixed images of TAMRA spectrum for Traz-TM-Q7 and Traz-TM, and the tumor to normal lung tissue ratio was calculated to investigate the specificity of each conjugate. *In vivo* HER2 receptor blocking studies were done by treating the 3T3/HER2+ tumor bearing mice with trastuzumab (5 mg, iv) one day before the Traz-TM-Q7 injection to confirm the specificity to HER2 receptor.

In addition, to investigate the sensitivity and specificity, Traz-TM-Q7 (50 $\mu$g/100 $\mu$L PBS) was intravenously injected to the mice that had both 3T3/HER2+ and Balb/3T3/HER2−/ZsGreen tumor nodules. The images were obtained one day after the Traz-TM-Q7 injection with the Maestro In-Vivo Imaging System in the same manner as described above using two filter sets. Immediately after finishing the fluorescence imaging study, the dissected lung specimens were frozen in Tissue-Tek (Sakura Finetek U.S.A., Inc., Torrance, CA) and stored at −80 °C. Frozen samples were sectioned with a cryostat microtome (CMI850UV, Leica Geosystems AG, Switzerland) at a thickness of 30 $\mu$m. Slides were analyzed under an Olympus BX61 microscope (Olympus America Inc., Center Valley, PA) equipped with the following filters: ZsGreen, excitation wavelength 470 to 490 nm, emission wavelength 515 nm long pass; TAMRA, excitation wavelength 530 to 570 nm, emission wavelength 590 nm long pass. Transmitted light differential interference contrast images were also acquired. Two-color composite images of fluorescence were generated using the standard software on the fluorescence microscope (MicroSuite Five, Olympus America Inc., Center Valley, PA).

## Results

**Quenching Capacity of Conjugates.** Quenching capacity was measured by adding 5% SDS, which dissociates the avidin tetramer into monomers. Figure 2(A) shows the change in fluorescence intensity of Av-TM-Q7 and Av-TM-Q7(CL) after SDS. The fluorescence intensity was fairly low without SDS for both Av-TM-Q7 and Av-TM-Q7(CL). After the incubation with 5% SDS, fluorescence intensities of Av-TM-Q7 increased 40-fold while minimal dequenching occurred with Av-TM-Q7(CL), the conjugate with cross-linked avidin.

For the antibody tagged conjugate quenching capacity was measured by adding 5% SDS and reducing with 2-ME, which dissociates the whole IgG into the heavy and light chains. The results with trastuzumab are shown in Figure 2(B). The fluorescence intensity of Traz-TM-Q7 was low, but was recovered after 5% SDS and 2-ME treatment with a 13-fold increase in light output. On the other hand, Traz-TM is unquenched and therefore fluorescent in PBS. The fluorescence intensity of Traz-TM was increased 3-fold with 5% SDS and 2-ME. Daclizumab conjugates showed similar results to the trastuzumab conjugates, 9-fold and 4-fold for Dac-TM-Q7 and Dac-TM, respectively. The 3−4-fold increased signal of both Traz-TM and Dac-TM with the treatment has been reported as the "Rhodamine effect", which is a small intrinsic activatable characteristic of the rhodamine family of fluorophores when conjugated with proteins and internalized into cells.[13] That is, Traz-TM-Q7 and Dac-TM-

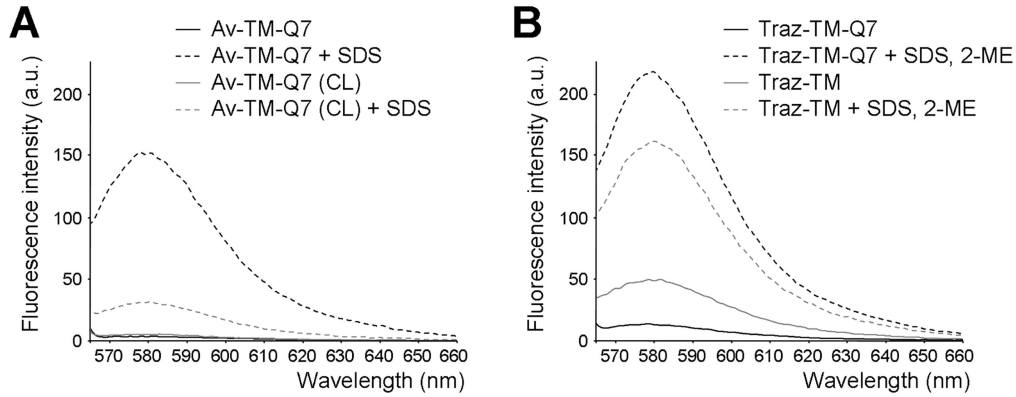


***Figure 2.*** *In vitro* fluorescent properties of the activatable conjugates using the FRET activation system. (A) Fluorescence emission spectra of Av-TM-Q7 and cross-linked, Av-TM-Q7(CL) at the same concentration (60 nM). The quenched fluorescence was increased 40-fold by denaturation of Av-TM-Q7. However, the fluorescence increased minimally in Av-TM-Q7(CL). (B) Fluorescence emission spectra of Traz-TM-Q7 and Traz-TM at the same concentration (75 nM). The quenched "activatable" probe, Traz-TM-Q7, increased 13-fold with denaturation. However, with the "always-on" probe, Traz-TM, the fluorescence increased minimally and its intensity in PBS was higher than that of Traz-TM-Q7.

Q7 were "activatable", whereas, Traz-TM and Dac-TM were "always-on".

**Activation of Quenched Conjugates after Internalization into the Target Cells.** In order to demonstrate activation of Av-TM-Q7 or Traz-TM-Q7 conjugates in the cell after internalization, *in vitro* cell experiments were performed using fluorescence microscopy. After incubation of SHIN3 cells with Av-TM-Q7 for 6 h, many fluorescent dots colocalizing to the endolysosomes were found in the cytoplasm of SHIN3 cells (Figure 3(A)). On the other hand, no fluorescence was observed with cross-linked Av-TM-Q7.

Both Traz-TM-Q7 and Traz-TM showed fluorescent signal on the surface of 3T3/HER2+ cells 1 h after incubation (Figure 3(B)). The fluorescence was observed inside the cell after 8 h of incubation with both conjugates, and the fluorescent dots were brighter for Traz-TM-Q7 than Traz-TM. Prior treatment with unlabeled trastuzumab diminished the fluorescence at both the 1 and 8 h time points. Fluorescence microscopy images were also obtained for Dac-TM-Q7 and Dac-TM; however, as expected, none of these images revealed surface or intracellular fluorescence.

***In Vivo*** **Fluorescence Imaging with Avidin Conjugates.** The *in vivo* images of Av-TM-Q7 and Av-TM-Q7 (CL) in SHIN3 tumor bearing mice are shown in Figure 4(A, B). The tumors in the peritoneal cavity were clearly imaged by Av-TM-Q7, whereas they could not be detected with the cross-linked conjugates. In the peritoneal membrane, submillimeter tumor nodules were detected by Av-TM-Q7, but the fluorescence was considerably lower with Av-TM-Q7 (CL).

To investigate the subcellular localization, Av-TM-Q7 or Traz-TM-Q7 was incubated with lysosomal marker (LysoTracker). As shown in Figure 3(C), the fluorescent dots from TAMRA were matched with lysosomal marker fluorescence.

***In Vivo*** **Fluorescence Imaging with Antibody Conjugates.** The results of imaging of 3T3/HER2+ lung tumor bearing mice are summarized in Figure 5(A, B). The whole body images demonstrated a low background for Traz-TM-Q7. For Traz-TM, however, nonspecific fluorescence could be observed throughout the field of view interfering with the ability to see the lung nodules. Trastuzumab pretreatment decreased the accumulation of Traz-TM-Q7 in the lung and tumor. The extracted lung images showed tumor fluorescence for Traz-TM-Q7. Traz-TM also accumulated in the tumor, but fluorescent signal was also observed in normal lung tissue. The tumor to normal lung tissue (background) ratios of Traz-TM-Q7 and Traz-TM were 22.4 ± 4.3 (AU) and 5.7 ± 1.8 (AU), respectively. The ratio was significantly higher in Traz-TM-Q7 than Traz-TM ($P < 0.01$, Mann−Whitney *U*-test).

Dac-TM-Q7 showed no fluorescent signal, whereas a generalized fluorescence signal was seen throughout the imaging field after Dac-TM had been injected into the mice. In *in vivo* experiments the fluorescence signal in the lung was considerably lower for Dac-TM-Q7 (due to lack of binding and internalization), however, generalized fluorescence was seen in mice injected with Dac-TM.

***In Vivo*** **Imaging in ZsGreen Transfected Tumor Bearing Mice.** To investigate the sensitivity and specificity of the conjugates for tumor detection, Av-TM-Q7 and Traz-TM-Q7 were tested in mice bearing ZsGreen transfected tumors. In the peritoneal SHIN3/ZsGreen tumor model, the fluorescent signal from TAMRA overlapped the signal from ZsGreen with almost complete concordance (Figure 6(A)). In the mice bearing lung metastases with both 3T3/HER2+ and Balb/3T3/HER2−/ZsGreen tumor in the lung, as expected there was no concordance between Traz-TM-Q7 enhancement of 3T3/HER2+ nodules and Balb/3T3/HER2−/ZsGreen nodules that do not express HER2 (Figure 6(B, C)).

## Discussion

Fluorophore−quencher pairs can be used to activate fluorescence for *in vivo* imaging. Activation takes place

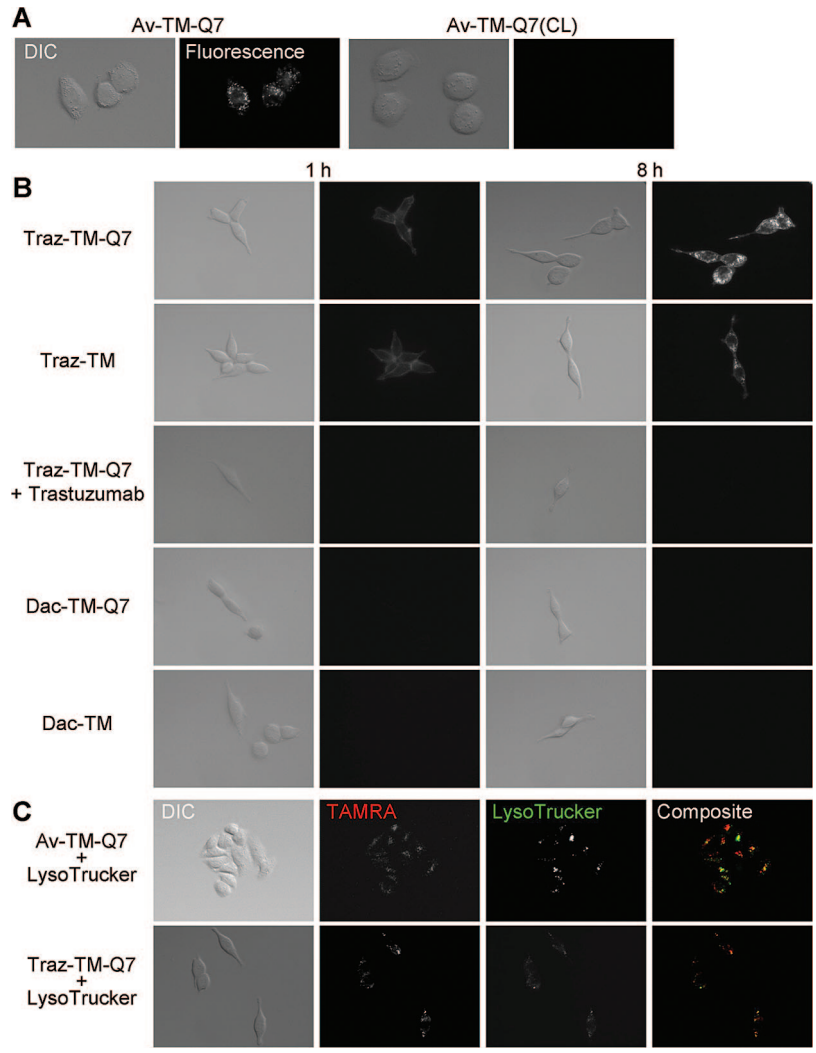


***Figure 3.*** Target specific FRET activation in tumor cells. Fluorescence microscopy (right) as well as differential interference contrast (left) images in SHIN3 cells (A) and 3T3/HER2+ tumor cells (B). (A) Fluorescent dots were observed within the cytoplasm 6 h after incubation with 10 $\mu$g/mL of Av-TM-Q7, whereas there was no fluorescence with Av-TM-Q7(CL) (10 $\mu$g/mL). (B) Both Traz-TM-Q7 and Traz-TM showed a fluorescent signal on the cell surface 1 h after incubation. Fluorescent dots were observed within the cytoplasm 8 h after incubation with both conjugates, however, the fluorescence was brighter for the "activatable" probe, Traz-TM-Q7. The non-HER2 ligand, daclizumab conjugates showed no binding and hence no fluorescence. (C) Av-TM-Q7 or Tra-TM-Q7 was incubated with LysoTracker in SHIN3 or 3T3/HER2+ cells, respectively. The fluorescent dots from TAMRA (red) were matched with the fluorescence from the lysosomal marker (green) for both activatable probes.

intracellularly, presumably by release of the quencher from the donor during lysosomal degradation of the carrier molecule. The quenching capacity of fluorophore−quencher pairs described here is substantially higher than previous self-quenching conjugates, in which multiple fluorophores of the same type quench each other. Therefore, conjugates with fluorophore−quencher pairs were much less fluorescent than self-quenched conjugates, leading to lower background signal and hence, higher TBR.

As we have shown, the fluorophore−quencher pair can be applied to a variety of targeting ligands. In this study, two tumor-specific targeting systems, one receptor−ligand based (D-galactose receptor vs avidin)[18] administered ip and one antibody−antigen based (human EGF receptor type2 (HER2) vs trastuzumab) administered iv, were employed demonstrating the flexibility of the approach. After binding to the target molecule and following endocytic internalization, these large polymeric proteins are generally separated into monomers and cut into small peptides or amino acids by oxidation/reduction, proteases, and/or pH-mediated degradation in the lysosome.[19] Avidin is a noncovalently bound homotetrameric glycoprotein, and it binds to D-galactose

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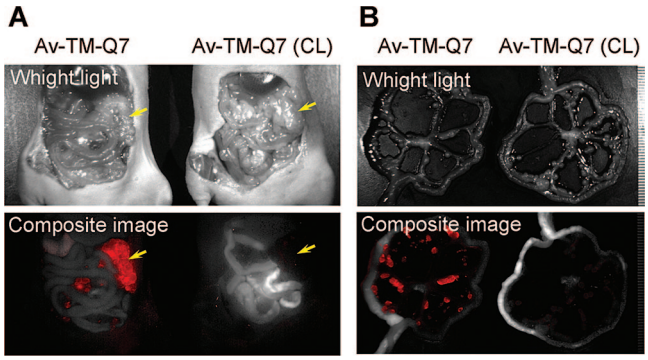


***Figure 4.*** *In vivo* imaging of D-galactose receptor-specific FRET activation. *In vivo* spectral fluorescence images of SHIN3 tumor bearing mice 3 h after intraperitoneal injection with Av-TM-Q7 or Av-TM-Q7(CL). The images were unmixed based on the spectral patterns of TAMRA, as well as autofluorescence, and composite images are shown (red, TAMRA; white, autofluorescence). (A) Spectral fluorescence image of the peritoneal cavities with Av-TM-Q7 clearly demonstrates disseminated tumor foci (yellow arrows). The signal from TAMRA was low for Av-TM-Q7(CL). (B) Close-up image of the peritoneal membranes demonstrates tumors as small as 1 mm with Av-TM-Q7. The signal for Av-TM-Q7(CL) was low and nondiagnostic.

receptor. D-Galactose receptor is commonly expressed on cancer cells such as ovarian cancer, colon cancer, gastric cancer, and pancreatic cancer, which have the potential to metastasize to the peritoneum.[20−22] Avidin is broken down into monomers in the lysosome, thus interfering with the fluorophore−quencher interaction. However, when avidin is covalently cross-linked, it resists degradation keeping the fluorophore−quencher pair intact.[2] The small increase in Av-TM-Q7(CL) might be due to imperfect cross-linking since the compound was not rigorously purified. Therefore, as shown in Figure 1, the quenched "off" state of the avidin−dye−quencher conjugate can be turned "on" once it is internalized into cancer cells. In contrast, the quenched "off" state of the cross-linked avidin−dye−quencher conjugate remains "off" even when bound to the receptor and internalized.

The use of monoclonal antibodies as targeting moieties has several advantages: (1) Many tumor specific antigens for which there are corresponding antibodies have been discovered. (2) These antigens can be used not only as diagnostic surrogate markers but also as therapeutic targets.[23−25] (3) Antibodies have a relatively "modular" design so that the chemistry needed for conjugation of one antibody

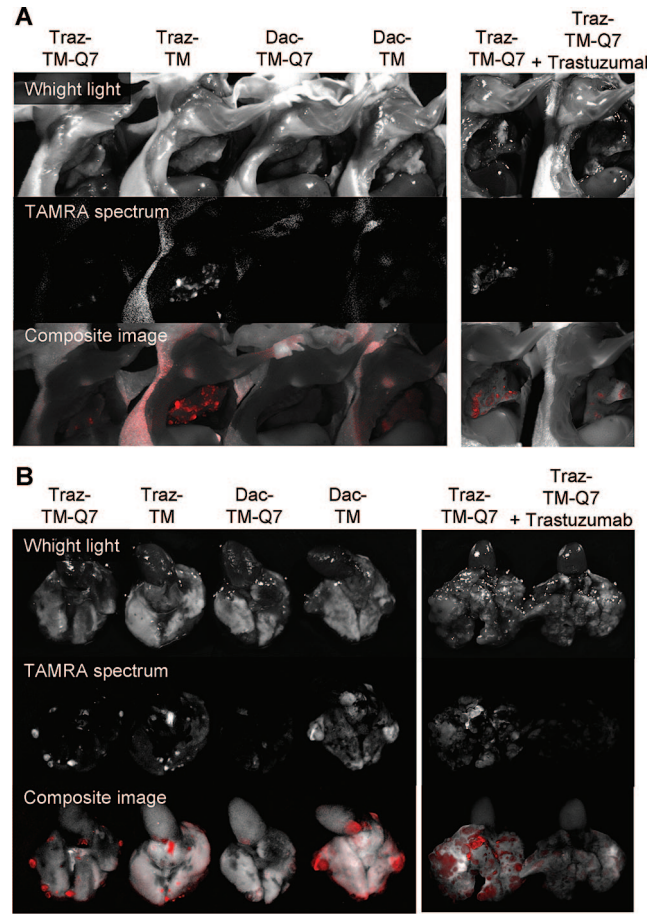


***Figure 5.*** *In vivo* imaging of HER2 receptor-specific FRET activation. *In vivo* spectral fluorescence images of 3T3/HER2+ tumor bearing mice were taken 1 day after intravenous injection with Traz-TM-Q7, Traz-TM, Dac-TM-Q7 or Dac-TM. The images were unmixed based on the spectral patterns of TAMRA as well as autofluorescence, and composite images are shown (red, TAMRA; white, autofluorescence). (A) The whole body images demonstrate the low background for the "activatable" probe, Traz-TM-Q7. The background signal was detected with the "always-on" probe, Traz-TM. Dac-TM-Q7 showed no fluorescent signal, whereas fluorescence signal could be seen in Dac-TM injected mice. Trastuzumab blocking decreased the accumulation of Traz-TM-Q7. (B) The extracted lung images demonstrates tumor fluorescence for both Traz-TM-Q7 and Traz-TM. However, fluorescent signal was also observed in normal lung tissue with the "always-on" probe, Traz-TM. The tumor as well as normal lung tissue was detected with the "always-on" nontargeted antibody conjugate, Dac-TM. The fluorescent signal was considerably lower for "quenched" probe Dac-TM-Q7.

to another moiety will likely be successful, with minor modification for a variety of antibodies (4) Antibodies have been engineered as variants with similar binding characteristics but different sizes (and hence, pharmacokinetics) such as Fab fragments and minibodies or diabodies.[26] (5) Antibodies by themselves can be therapeutic reagents. Therefore, imaging with monoclonal antibodies can play important roles

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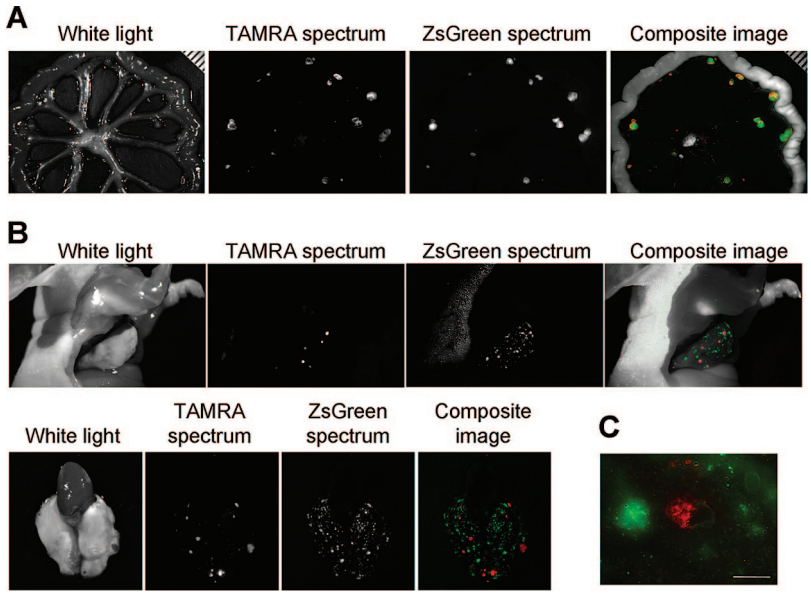


***Figure 6.*** Specificities of Av-TM-Q7 and Traz-TM-Q7 to the target tumors *in vivo*. (A) Close-up images of peritoneal membrane of the SHIN3/ZsGreen tumor bearing mice demonstrate the coincidence of TAMRA fluorescent signal (red) and constituitively expressed ZsGreen fluorescent signal (green). (B) In the mice with both 3T3/HER2+ and Balb/3T3/HER2−/ZsGreen tumor, the signal from TAMRA (red, 3T3/HER2+ cells) did not overlap with ZsGreen (green, Balb/3T3/HER2−/ZsGreen cells). (C) Fluorescence microscopic image of lung slice demonstrates the absence of overlap between TAMRA and ZsGreen (bar = 200 $\mu$m).

in therapeutic decision making as well as tumor detection. A number of humanized monoclonal antibodies have already received clinical approval as human cancer therapies.[27−30] Among these, trastuzumab (Herceptin, Genentech Inc.) was selected for testing with the fluorophore−quencher system. Trastuzumab is a monoclonal antibody which binds to human epidermal growth factor receptor type 2 (HER2) expressed on the cell surface of some tumors. Trastuzumab binds to HER2 on the plasma membrane and subsequently, is gradually internalized into the cytoplasm whereupon it is degraded in the lysosome.[31] The fluorescence of the trastuzumab−dye−quencher conjugate is quenched while the antibody is outside of the cell, but when the conjugate is internalized, it is dequenched and activated (Figure 1).

*In vitro* microscopic studies demonstrated fluorescent dots within SHIN3 cells exposed to Av-TM-Q7. The many bright fluorescent intracellular dots indicate that Av-TM-Q7 was internalized within the endoplasmic vesicles in the cytoplasm.[2,32] On the other hand, the fluorescence intensity in the cell was fairly low for the cross-linked avidin conjugate. This observation supports the hypothesis that the degradation of avidin is responsible for activating the fluorescence of the fluorophore−quencher pair. As shown in Figure 4, the activation of Av-TM-Q7 was also demonstrated by *in vivo* studies. The SHIN3 tumor was only detected with Av-TM-Q7, not with cross-linked conjugates. In addition, the TAMRA fluorescence showed perfect coincidence with the ZsGreen fluorescence of SHIN3/ZsGreen tumors (Figure 6(A)). These results demonstrate that D-galactose receptor positive tumors can be visualized effectively *in vivo* with Av-TM-Q7. This method could be useful for detecting small cancers in the abdominal cavity during laparoscopy. However, avidin is immunogenic in humans, and therefore another targeted agent based on a human or humanized protein and employing site-specific conjugation chemistry for the fluorophore−quencher pairs must be developed.

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Our results show that trastuzumab, an FDA-approved monoclonal antibody, can be used as a tumor targeting protein for fluorophore−quencher pairs. The bright fluorescent intracellular dots observed in 3T3/HER2+ cells exposed to Traz-TM-Q7 for 8 h were brighter than those exposed to Traz-TM, although the brightness was similar when the conjugates were on the surface of the cell after 1 h incubation. That is, the "always-on" conjugate, Traz-TM, was bright both outside and inside of the cell; on the other hand, the "activatable" conjugate, Traz-TM-Q7, was only bright inside of the cell. In addition, excess trastuzumab blocked Traz-TM-Q7 binding while incubation with Dac-TM-Q7 and Dac-TM did not yield fluorescence in the same cells. These observations indicate that the fluorescent signal from Traz-TM-Q7 is specific to cells expressing the HER2 receptor and that the fluorophore−quencher interaction system is effective in these cells. However, there was a low intensity fluorescence arising from Traz-TM-Q7 on the surface of HER2+ cells. The reason for activation of the molecule at the cell surface is uncertain but might be related to the environmental changes (e.g., change in hydrophobicity due to localizing adjacent to the cell membrane) induced by protein−protein interactions, leading to partial release of fluorophore−quencher pairs. The observation that Dac-TM-Q7, which does not bind to HER2, did not show fluorescence even on the surface supports this hypothesis.

An important measure of the success of an optical probe for *in vivo* imaging is its TBR. As shown in Figure 5, the background fluorescence was lower in the "activatable" conjugate, Traz-TM-Q7, than in the "always-on" conjugate, Traz-TM, *in vivo*. In addition, the non-HER2 targeted antibody, Dac-TM, showed substantial background fluorescence while the quenched Dac-TM-Q7 injected mice had no fluorescence. The measured tumor-to-background ratio was significantly higher in "activatable" probes Traz-TM-Q7 than Traz-TM ($p < 0.01$), indicating that FRET dequenching is a very effective mechanism of activating fluorescent probes. The fluorescent signal from Traz-TM-Q7 was specific to HER2 receptor *in vivo*, since prior blocking with trastuzumab diminished the fluorescence in the tumor nodules and there was no overlap between HER2 positive tumors and ZsGreen transfected HER2 negative tumors (Figure 6(B)).

Optical imaging represents a powerful technology for cancer research. The study of cancer biology *in vivo* through expression and imaging of endogenously expressed fluorescent proteins and the development of clinical applications for the treatment of cancer especially for florescence-guided conventional or endoscopic/laparoscopic surgery (video in Supporting Information) with disease-specific fluorescent probes are currently two major applications of *in vivo* cancer imaging. Early studies involving imaging of fluorescent proteins revealed the power of optical imaging in cancer biology, such as for the imaging of cancer invasion and metastasis in longitudinal studies.[33] One limitation to the use of fluorescence proteins for cell labeling in humans is that this process necessitates gene transfection,[34] which is typically achieved in animal models after viral transfection.[35] Small fluorescent or quenching molecules such as organic fluorophores and quenchers used in this study, however, may be used similarly to conventional contrast agents for nuclear medicine or MRI and are therefore better-suited for development of targeted optical probes for clinical applications as they do not require manipulation of the host genome. Additionally, fluorophores and quenchers are smaller than fluorescent proteins and easily conjugated to targeting proteins such as antibodies.

In conclusion, the proposed activation system based on a FRET pair resulted in images with low background and high activation at the target leading to diagnostic *in vivo* imaging of *in situ* tumors. This method may be widely applicable to other tumor targeting strategies and may have important translational implications to the fluorescence-guided endoscopy or surgery.

**Acknowledgment.** This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

**Supporting Information Available:** Av-TM-Q7 (120 $\mu$g) was injected intraperitonealy into the SHIN3 tumor bearing mouse, and real time surgery was performed. The video was taken 3 h after the injection using green filter sets (excitation, 510 to 550 nm; emission, 570 nm long pass) with FluorVivo (INDEC Biosystems). The target tumor was identified in orange and easily resected as shown. This material is available free of charge via the Internet at http://pubs.acs.org.

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